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Received: 7 February 2025 | Accepted: 28 March 2025

DOI: 10.1002/ar.25673

REVIEW ARTICLE

The Anatomical Record | American Association for Anatomy | WILEY

Neutrophil extracellular traps mediate pathophysiology of hepatic cells during liver injury

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Abstract

Neutrophil extracellular traps (NETs) are web-like, bactericidal structures produced by neutrophils and are composed principally of extracellular DNA, histones, elastase, and myeloperoxidase, among other components. NET formation is an innate immune response that is beneficial for pathogen killing and clearance. However, excessive NET formation and clearance defects can lead to inflammation and induce damage to host organs. NETs are also implicated in the development of noninfectious inflammatory disorders, such as liver injury in chronic liver diseases. The liver parenchyma contains hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells. Each of these cells possesses unique structures and functions, and their interactions with NETs result in pathophysiological changes contributing to liver injury. This review updates the findings related to the modes of action and molecular mechanisms by which NETs modulate the pathophysiology of various hepatic cells and potentiate liver injury. The article also reviews the roles of NETs in hepatic ischemia reperfusion injury, hepatocellular carcinoma pathogenesis, and cancer metastasis. Last, we examine data to determine whether NETs induce crosstalk among various hepatic cells during liver injury and to identify future research directions.

KEYWORDS

cancer metastasis, hepatocellular carcinoma, ischemic reperfusion injury, neutrophil extracellular traps, pathophysiology of hepatic cells

1 | INTRODUCTION

First reported in 2004, neutrophil extracellular traps (NETs) are web-like structures produced by neutrophils (Brinkmann et al., 2004). As depicted in Figure 1, NETs are composed principally of extracellular DNA decorated with histones, neutrophil elastase (NE), and myeloperoxidase (MPO), as well as other antimicrobial molecules that kill pathogens (Brinkmann, 2018; Brinkmann et al., 2004). The primary function of NETs is to trap and kill pathogens (Brinkmann et al., 2004; Kolaczkowska et al., 2015; McDonald et al., 2012; Urban et al., 2009). NETs formation is considered to be a component of the innate immune response but is also involved in the regulation of adaptive immunity (Liew & Kubes, 2019; Tillack et al., 2012; Zhu et al., 2023). Pathologic or excessive NET generation causes pro-inflammatory immune damage to host organs. Taken together, the antibacterial and pro-inflammatory activity of NETs underscore the double-edged sword of innate immunity (Kaplan & Radic, 2012)—this duality is reminiscent of Dr. Jekyll and Mr. Hyde (Lauková & Konecná, 2018). Furthermore, NETs have been implicated in the development of a multitude of noninfectious inflammatory diseases,

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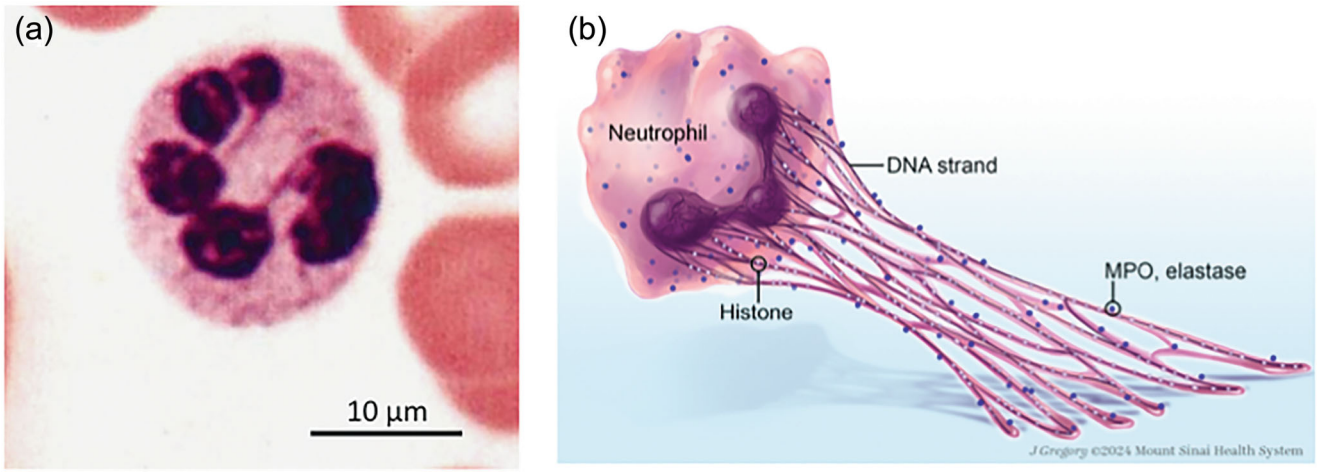

FIGURE 1 (a) Neutrophil as seen in a blood smear, Giemsa stain. This is a mature neutrophil showing a multi-lobulated nucleus consisting of 5 lobes connected by thin strands of nuclear material. The cytoplasm is lightly stippled with purplish granules. The pale halo in the center represents the Golgi apparatus. (b) Drawing of neutrophil extracellular traps (NETs). The traps emerging from the neutrophil are networks of extracellular fibers, consisting of extracellular DNA, neutrophil myeloperoxidase (MPO), neutrophil elastase, and citrullinated histones, among others. *Source*: Courtesy of J Gregory, Associate Director of Instructional Design, Mount Sinai Health System.

discussed below (Brinkmann, 2018; Jorch & Kubes, 2017; Papayannopoulos, 2018; Zhao & Jin, 2022).

The liver is the largest and major digestive organ of the body. It was at one time considered to be the seat of life; hence the name liver—the thing we live with. NETs are highly implicated in liver injury during ischemia reperfusion injury (IRI) following liver transplantation, endotoxin-induced liver injury, excessive inflammation in sepsis, chronic liver disease pathogenesis, such as alcoholic liver disease (ALD), metabolic dysfunction-associated steatotic liver disease (MASLD—previously-named NAFLD), metabolic dysfunction-associated steatohepatitis (MASH—previously named NASH), hepatocellular carcinoma (HCC), and metastasis. The liver parenchyma comprises four major cell types: hepatocytes, liver sinusoidal endothelial cells (LSECs), Kupffer cells, and hepatic stellate cells (HSCs). Each cell type possesses unique structures and functions, and their interactions with NETs result in pathophysiological changes that contribute to liver injury. Neutrophils per se are known to interact with hepatic cells to promote various forms of liver damage (Huang et al., 2023). The focus of this article is to review data and findings related to the modes of action and molecular mechanisms characterizing how NETs modulate the pathophysiology of LSECs, Kupffer cells, HSCs, and hepatocytes, which lead to various forms of liver damage, including ischemic reperfusion, tumorigenesis, and cancer metastasis. An overview of these processes is schematically represented in Figure 2. As an introduction, we highlight the historical discovery of NETs, the structure of NETs and their molecular components, forms of NETosis, bioactive molecules mediating NETosis, functions of NETs, and

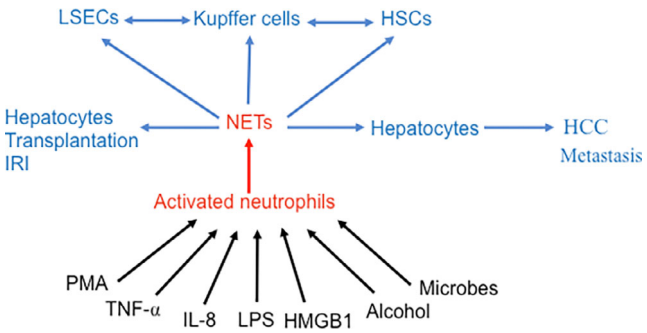

FIGURE 2 Schematic representation of various stimuli that activate neutrophils, provoking the production of neutrophil extracellular traps (NETs). This, in turn, modulates the pathophysiology of hepatic cells. Potential crosstalk between Kupffer cells and liver sinusoidal endothelial cells (LSECs) or hepatic stellate cells (HSCs) is indicated by the double-headed arrows. NET elicitation of liver injury associated with liver ischemia reperfusion injury (IRI) following transplantation, and hepatocellular pathogenesis and cancer metastasis are also included in the schema. HMGB1, high mobility group B1; LPS, lipopolysaccharide; PMA, phorbol myristate acetate.

NETs-associated diseases. We also examine data to determine whether NETs induce crosstalk among various hepatic cells during liver injury and to identify future research directions.

2 | WHAT ARE NETS

NETs are extracellular web-like fibers released from neutrophils. By transmission and scanning electron microscopy, NETs are observed to contain smooth fibers (also

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described as threads or filaments) with a diameter of 15–17 nm and attached globules of around 25 nm in diameter (Brinkmann et al., 2004; Brinkmann & Zychlinsky, 2007). Several fibers can be wound into larger bundles of fibers (or cables) up to 100 nm in diameter. These cables appear to form a complex of three-dimensional structures resembling spider webs and covering areas of several square μm. The fibers represent a chain of nucleosomes of decondensed chromatin that form the backbone of the NETs, as they can be degraded by DNases but not by proteases (Brinkmann & Zychlinsky, 2007; Buchanan et al., 2006). Atomic force microscopy revealed that the DNA filaments are organized into a branching of networks decorated with antimicrobial and cytoskeletal proteins and proteases (Pires et al., 2016; Santocki & Kolaczkowska, 2020). The DNA-branching filaments form a mesh creating fenestrae measuring around 0.05 μm which is commensurate with the size of small pathogens.

NETs are composed principally of decondensed chromatin (extracellular DNA), citrullinated nuclear histones (cit-H3, H2A, and H4), granular NE, MPO, and cathepsin G derived from primary azurophilic granules. There is also lactoferrin derived from specific (secondary) granules. Gelatinase, lysozyme C, and antimicrobial peptides like cathelicidins and defensins derived from tertiary granules are also present (Brinkmann, 2018; Brinkmann et al., 2004; Brinkmann & Zychlinsky, 2012; Papayannopoulos, 2018). In addition, cytoplasmic calprotectin with anti-fungal activity was identified (Sollberger et al., 2018; Urban et al., 2009). Moreover, cytoskeletal protein actin and glycolytic enzymes have been reported to be present in NETs (Urban et al., 2009). Other constituents include high mobility group B1 (HMGB1), proteinase 3, IL-37, heat shock proteins, MMP-9, and MMP-2 (Babuta et al., 2024; Huang et al., 2015; Santocki & Kolaczkowska, 2020; Tohme et al., 2016; Wang et al., 2024). The positively charged proteins are attached to the negatively charged DNA by electrostatic forces. In total, at least 20–30 different proteins have been identified in NETs. Their presence depends on whether phorbol myristate acetate (PMA), cytokine IL-8, or microbes were employed as inducers (Brinkmann & Zychlinsky, 2012; Santocki & Kolaczkowska, 2020), rather than differences in neutrophil physiology (Wang et al., 2024). Moreover, different diseases may exhibit varying protein profiles in NETs. Accordingly, the differences in NET composition could impact the NET's overall functions. While the majority of DNA in NETs is derived from the nucleus, they also contain a small amount of mitochondrial DNA (Yousefi et al., 2009).

NETs are released by neutrophils in a process known as NETosis. The term NETosis or beneficial suicide (Brinkmann & Zychlinsky, 2007) was originally intended to indicate the death of neutrophils a few hours after releasing NETs (Brinkmann & Zychlinsky, 2012; Jorch & Kubes, 2017). However, pathogens are able to trigger a rapid and vital form of NETosis, in which neutrophils are still viable to perform their phagocytic and other functions after producing and releasing NETs (Yipp et al., 2012).

To date, two forms of NETosis have been established: the lytic/suicidal pathway and non-lytic/vital NETosis. In the lytic/suicidal pathway, rupture of the neutrophil plasma membrane is needed. In the non-lytic/vital NETosis, the neutrophil plasma membrane remains intact. These forms of NETosis are further described as follows.

2.1 | Lytic/suicidal NETosis

Several pathways are involved in the formation of lytic NETs. Activation of NADPH oxidase triggers reactive oxygen species (ROS) generation in neutrophils, leading to activation of peptidylarginine deiminase 4 (PAD4), a nuclear enzyme (Denning et al., 2019; Fuchs et al., 2007; Wang et al., 2009). Subsequently, PAD4 converts arginine residues on histones into citrulline, resulting in chromatin decondensation. This leads to NET formation (Li et al., 2010; Sollberger, 2018; Van Avondt & Harti, 2018; Wang et al., 2009). Further, neutrophil NE and MPO are activated and translocated to the nucleus, further promoting chromatin decondensation (Jorch & Kubes, 2017; Papayannopoulos et al., 2010). NE can also degrade actin filaments and block the phagocytosis pathway (Metzier et al., 2014; Papayannopoulos, 2018). Thereafter, the nuclear membrane is disrupted, allowing single-stranded DNAs and histones released into the cytoplasm to be decorated with antibacterial proteins (citH3, H2A, and H4, NE, MPO, and cathepsin G) and cytosolic proteins (calprotectin) to form early NETs. Finally, NETs are released through plasma membrane disruption into the extracellular space (Fuchs et al., 2007). Neutrophils subsequently die in a manner that is morphologically distinct from necrosis or apoptosis. Lytic NETosis typically occurs in 2–4 hours. Lytic NETosis can be induced by different stimuli such as PMA, auto-antibodies, or cholesterol crystals. In vitro studies revealed that neutrophils generate NETs in response to treatment with PMA, lipopolysaccharide (LPS) derived from gut gram-negative bacteria, TNF-α, and IL-8 (Brinkmann et al., 2004; Hakkim et al., 2011; Kenny et al., 2017; Saffarzadeh et al., 2012).

2.2 | Non-lytic/vital NETosis

In viable NET formation, PAD4 is activated by TLR-2 and TLR-4 receptors on neutrophils under different

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stimuli. For example, bacterial LPS exposure of neutrophils results in the entry of PAD4 into the nucleus to citrullinate histones, H3 and H4, thereby decondensing/unfolding chromatin DNA strands (Li et al., 2010; Wang et al., 2004; Wang et al., 2009). In contrast to the formation of lytic NET, the PAD4 gene is activated independent of NADPH activity and ROS generation. Translocation of unfolded chromatin from the nucleus into the cytoplasm via nuclear membrane blebbing occurs without disruption of the membrane. In addition, unlike lytic NETosis, the non-lytic formation of NETs induced by bacteriostatic proteins occurs in the form of vesicles (Jorch & Kubes, 2017; Yipp & Kubes, 2013). Subsequently, the vesicles and the contents are released from the cell into the extracellular space by exocytosis without disruption of the plasma membrane (Van Avondt & Harti, 2018). During the process, the neutrophils are viable and not lysed, and despite the absence of nuclear DNA, the anucleated neutrophils are still capable of many key neutrophil functions, including crawling, chemotaxis, phagocytosis, and production of oxidants to kill ingested pathogens in vivo (Yipp et al., 2012). In contrast to lytic NETosis, the non-lytic NETosis is rapidly induced within 5–60 min. This form of NETosis can be induced by LPS, *Staphylococcus aureus* (*S. aureus*) through both complement receptors and TLR2 ligands or elicited by *Escherichia coli* (*E. coli*) directly via TLR4 or indirectly via TLR4-activated platelets (Jorch & Kubes, 2017).

To summarize, lytic NETosis is provoked by PMA, LPS, TNF-α, and IL-8. The NETosis is ROS-dependent, and the neutrophils die. Non-lytic NETosis is induced by LPS, *S. aureus*, and *E. coli*. This form of NETosis is ROS-independent, and the neutrophils retain their viability.

2.3 | Functions of NETs and associated diseases

NETs have a wide range of efficacy against bacteria, fungi, viruses, and parasites (Brinkmann & Zychlinsky, 2012). NETs were first described as a mechanism to enable neutrophils to trap and kill pathogens. Indeed, inhibition of NET formation causes an increased susceptibility to bacterial infection in both mice and humans. NE, a granular protein, can also degrade certain bacterial virulence factors (Weinrauch et al., 2002). Owing to the intricate web-like fibrous structures, NETs may provide a high local concentration of antimicrobial molecules acting against entrapped microbes. This also serves as a physical barrier to entrapped microbes, preventing their further spread (Brinkmann & Zychlinsky, 2007; Brinkmann & Zychlinsky, 2012). Although the components of NETs possess bactericidal activity, excessive NET formation and clearance defects can lead to inflammation and immune damage to host organs. The NET components can modulate the immune response by interacting with other immune cells, notably macrophages, liver-resident Kupffer cells (Bukong et al., 2018; Farrera and Fadeel, 2013), and LSECs (McDonald et al., 2013). By interacting with HSCs, NETs promote hepatic fibrogenesis (Babuta et al., 2024; Xia et al., 2025).

In addition to contributing to infectious diseases, NETs have also been implicated in the development of a multitude of noninfectious inflammatory diseases (Brinkmann, 2018; Jorch & Kubes, 2017; Liu, Yan, et al., 2022; Papayannopoulos, 2018; Sørensen & Borregaard, 2016; Wang, Kim, et al., 2024; Zhao & Jin, 2022). These include sterile inflammatory diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis, atherosclerosis, thrombosis, vasculitis, diabetes, cystic fibrosis, pulmonary COPD, hepatic ischemia reperfusion injury (IRI), cancers of various organs, as well as tissue injury and wound healing. More exciting findings showed the association of NETs with liver metabolic disorders such as alcoholic hepatitis, MASLD, and MASH. NETs are also linked with the progression of these pathologies to cirrhosis and ultimately hepatocellular carcinoma (HCC) (Fa et al., 2023; Liu, Zhang, et al., 2022; Minciuna et al., 2024; van der Windt et al., 2018; Zhu et al., 2023). In addition, excess NET formation is associated with the pathology of inflammatory bowel diseases, namely, ulcerative colitis and Crohn's disease (Honda & Kubes, 2018).

Having introduced an overview of the NET structures, formation, functions, and associated diseases, we now discuss the actions of NETs on the pathophysiology of LSECs, Kupffer cells, HSCs, and hepatocytes. Figure 3 shows the distribution of liver cells in the liver lobule.

3 | LIVER SINUSOIDAL ENDOTHELIAL CELLS

LSECs are specialized endothelial cells that line the liver sinusoids. They are highly fenestrated and lack a basement membrane, providing an efficient filtering function and bidirectional exchange of molecules between sinusoidal blood and hepatocytes (Mak & Shin, 2021). LSEC interactions with NETs play significant roles in health and disease. Healthy interactions between LSECs and NETs support endothelial barrier functions, promoting proper blood flow and nutrient exchange. On the other hand, in conditions such as chronic hepatitis, liver fibrosis, and cirrhosis, persistent inflammation will lead to excessive NET formation. This can damage LSECs,

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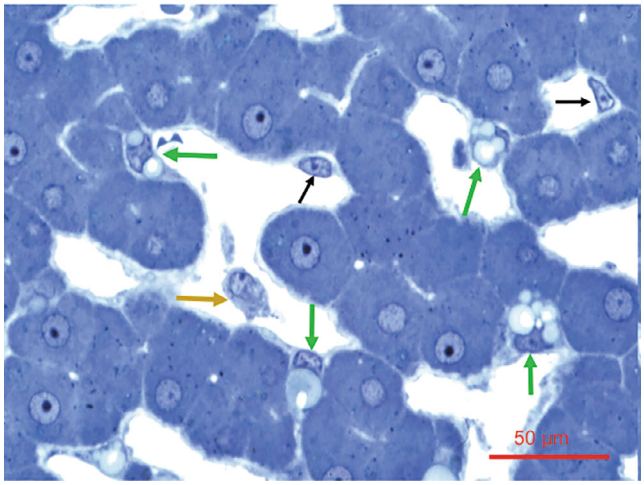

FIGURE 3 Liver cell types. Liver sinusoidal endothelial cells are indicated by black arrows. The brown arrow indicates a Kupffer cell in the sinusoidal lumen. Green arrows mark hepatic stellate cells that show distinct cytoplasmic lipid droplets (lightly greenish), a morphological marker of HSCs. Hepatocytes are stained blue. Plastic section, toluidine blue stain.

disrupting the endothelial barrier and further exacerbating liver injury. In addition, NET-induced endothelial activation can lead to increased expression of adhesion molecules and coagulation factors, promoting thrombosis formation.

3.1 | Formation of NETs in liver sinusoids during sepsis—Intravital microscopy analysis

In mice, neutrophils migrate to the liver sinusoids during endotoxemia and sepsis induced by LPS derived from *E. coli*. By intravital microscopy, neutrophils were detected releasing NETs into the sinusoidal lumen to entrap bacteria in the bloodstream, thus preventing their dissemination (McDonald et al., 2012). During sepsis, NETs increase bacterial trapping four times greater than that of Kupffer cells/macrophages. NET production requires platelet–neutrophil interaction, which is mediated by TLR4 signaling (Clark et al., 2007). NETs are able to ensnare circulating bacteria to provide intravascular immunity, thus protecting against bacterial dissemination during septic infections.

In another study, intravital multiphoton microscopy imaging demonstrated an increase of NETs within the sinusoids during the sepsis in mice induced by LPS (Tanaka et al., 2014). The NETs aggregate with platelets, occluding the hepatic microcirculation. This slows down blood flow in the sinusoids, which causes an adverse effect of intravascular NETs on the host during sepsis. Subsequently, NETs facilitate the detachment of sinusoidal endothelial cells, resulting in extravasated platelet aggregation in the space of Disse, contributing to sepsis-induced liver dysfunction (Sakurai et al., 2017).

MRSA (methicillin-resistant *S. aureus*) infection in mice induces NET formation in the liver. Intravital imaging microscopy (Kolaczkowska et al., 2015) revealed localization of NETs in the liver sinusoids, anchoring to LSECs, in part, via von Willebrand factor. The formation of NETs was most robust at 4 and 8 h and persisted beyond 24 h post infection. Moreover, the NETs exhibited high NE activity. DNase treatment was effective at removing extracellular DNA but less effective in inactivating the NE activity of NETs. By contrast, inhibition of NET production in PAD4-deficient mice or abolishment of the proteolytic activity in NE-deficient mice prevented host liver damage.

3.2 | Interactions between LSECs and neutrophils during liver injury—Signaling molecules in NET production

The interplay between LESCs and neutrophils leading up to neutrophil activation and the consequence of NET formation in the liver involves a series of sequential events. These include neutrophil recruitment to liver sinusoids, neutrophil adhesion to LSECs, neutrophil transmigration through the sinusoidal wall, and subsequently, activation of neutrophils to produce NET formation in the space of Disse. These are described below and outlined in Figure 4.

During the course of liver injury/diseases, circulating neutrophils are recruited to the liver sinusoids in response to the secretion of a multitude of chemoattractants by LSECs, such as CXCL1, MCP-1 (macrophage monocyte chemoattractant protein-1), IL-8, among others (Wang & Liu, 2021). Neutrophils in the sinusoids can adhere to the sinusoidal endothelium via the activation of TLR4 on LSECs, inducing the deposition of serum-derived hyaluronan-associated protein (SHAP) (McDonald et al., 2013). The hyaluronic acid then engages CD44 on neutrophils, facilitating the adhesion of neutrophils to LSECs. During the transmigration through the sinusoidal wall, LSECs and neutrophils further interact through adhesion molecule ICAM-1 expression on endothelial cells and β2- integrin (CD11/CD18 [LFA-1, Mac-1]) on neutrophils (Gujral et al., 2004). In addition, vascular cell adhesion molecule-1 (VAMC-1) and its receptor also contribute to the trans-endothelial migration process (Essani et al., 1997; Jaeschke, 2003). Interestingly, when LSECs are injured by galactosamine/endotoxin treatment in mice, large gaps can be observed

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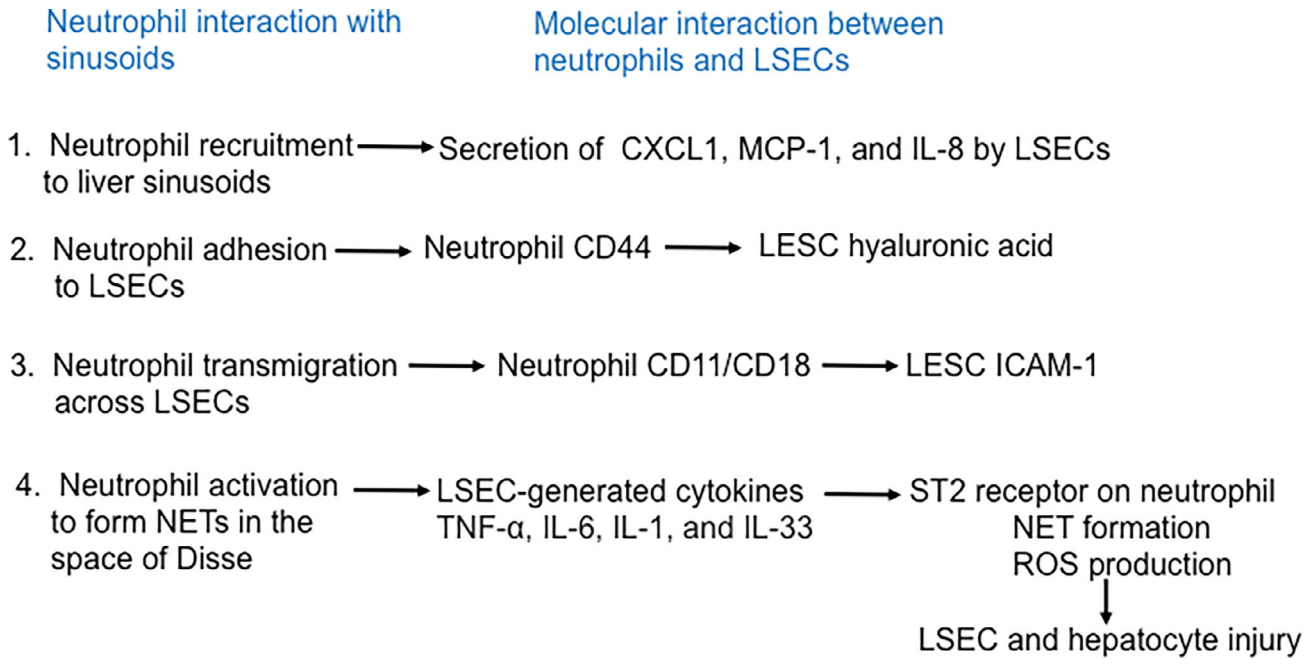
Neutrophil interaction with sinusoids
Molecular interaction between neutrophils and LSECs
1. Neutrophil recruitment to liver sinusoids
Secretion of CXCL1, MCP-1, and IL-8 by LSECs
2. Neutrophil adhesion to LSECs
Neutrophil CD44
LESC hyaluronic acid
3. Neutrophil transmigration across LSECs
Neutrophil CD11/CD18
LESC ICAM-1
4. Neutrophil activation to form NETs in the space of Disse
LSEC-generated cytokines TNF-α, IL-6, IL-1, and IL-33
ST2 receptor on neutrophil
NET formation
ROS production
LSEC and hepatocyte injury

FIGURE 4 Interactions between neutrophils and LSECs during liver injury. These occur in four sequential steps: (1) LSECs produce various chemokines to recruit neutrophils to the sinusoid; (2) neutrophils adhere to LSECs through interaction between neutrophil CD44 and LESC hyaluronic acid; (3) neutrophils and LSECs are further interacted via CD11/CD18 on neutrophils and adhesion molecules ICAM-1 on LSECs to facilitate neutrophil transmigration across the sinusoidal endothelium; and (4) neutrophils are activated in the space of Disse to produce NETs and ROS through interaction between LSEC-generated cytokines and ST2 receptors on neutrophils. This results in LSECs and hepatocyte injury.

on these endothelial cells with neutrophils occupying the openings of the endothelial gaps (Ito et al., 2006). These gaps permit neutrophils to come into direct contact with apoptotic hepatocytes, facilitating neutrophil transmigration through the sinusoidal endothelium. It was speculated that hepatocyte apoptosis in IL-8 galactosamine/endotoxin injured mice could deliver an intense signal for trans-endothelial migration of primed neutrophils accumulated in the liver sinusoids (Lawson et al., 1998). Yet the precise mechanisms behind this remain unclear.

It has been shown that neutrophil CD11b/CD18 engagement with ICAM during neutrophil migration through the sinusoidal wall also triggers robust ROS production via NADPH oxidase (Jaeschke, 2000, 2003). These bioactive molecules are capable of inflicting injury to LSECs, parenchymal hepatocytes, and perisinusoidal HSCs in the space of Disse. Inflamed and activated LSECs also secrete increased amounts of cytokines TNF-α, IL-6, IL-1, and IL-33 (Yazdani et al., 2017) and chemokines CXCL1 and CXCL2 (Connolly et al., 2010). TNF-α is well known to be a strong activator for neutrophils (Gupta et al., 2010; Ito et al., 2006). IL-33 is a DAMP (damage-associated molecular pattern) molecule (Denning et al., 2019) mainly secreted by LSECs. It drives neutrophil activation and NET generation through neutrophil receptor ST2 (suppression of tumorigenicity 2) as seen during liver ischemia reperfusion. This process causes excessive sterile inflammation and liver injury (Yazdani et al., 2017). In addition, NETosis can directly damage LSECs, as well as other types of endothelial cells such as HUVECs (human umbilical vascular endothelial cells). This is elaborated on in Section 3.3.

3.3 | NETs directly induce endothelial cell damage and the role of histones

NET formation in neutrophils can be induced by co-culture of neutrophils and activated endothelial cells derived from HUVECs, which is partially dependent on the production of IL-8, a neutrophil chemo-attractant factor (Gupta et al., 2010). Prolonged culture for 18 h, however, resulted in endothelial cell damage, which could be abrogated by diphenyleneiodonium (DPI) inhibition of the NADPH oxidase pathway that is required for NETosis, or by disruption of NETs with DNase1. In another study, NETs were isolated and prepared from human neutrophils stimulated with PMA (Saffarzadeh et al., 2012). Addition of the NETs to the culture of HUVECs was found to induce injury of endothelial cells, likely mediated by MPO. Similarly, the addition of histones to endothelial cell culture caused endothelial cell death,

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which was reduced by pre-incubation of endothelial cells with antibodies against histones (Xu et al., 2009). Yet the pathogenic relevancy for distinct organ system injury remains to be defined. Together, these data imply that activated endothelium induces the formation of NETs that are toxic to endothelial cells, thereby creating a vicious cycle that results in more cellular damage.

3.4 | NETs and LESCs interactions contribute to portal hypertension

LSECs express chemo-attractant CXCL1 in response to the induction of mechanical stretch in mice (Hilscher et al., 2019). Subsequently, neutrophils are recruited by CXCL1 to the liver sinusoids and become activated with the generation of NETs. This induces aggregation of microthrombi composed of platelets, fibrin, and erythrocytes, promoting portal hypertension through pressure and space effects in the liver sinusoids, reminiscent of cirrhosis.

Collectively, healthy interactions between LSECs and NETs support endothelial barrier functions, promote proper blood flow, and facilitate nutrient exchange. On the other hand, persistent inflammation will elicit excessive NET formation. Histones and NE from NETs can injure LSECs, disrupting the endothelial barrier and aggravating liver injury. NET-induced endothelial activation increases the expression of adhesion molecules and coagulation factors, promoting thrombosis formation and portal hypertension. Elucidation of the complex molecular mechanisms involved in neutrophil recruitment, adhesion, and transmigration across the sinusoidal endothelium leading up to NET formation provides an opportunity for intervention of liver injury induced by NETs. Nevertheless, there is scarcely any study examining the impacts of NETs on the capillarization of the liver sinusoids, which is a critical pathology producing hepatic dysfunctions (Mak et al., 2022).

4 | KUPFFER CELLS/ MACROPHAGES AND NETS INTERACTIONS

Kupffer cells are liver-resident macrophages that reside in the lumen of the sinusoids and make up 30% of the hepatic non-parenchymal cell population. In addition to Kupffer cells, there are also monocyte-derived "infiltrating" macrophages in the liver (Krenkel & Tacke, 2017). Most investigations consider Kupffer cells and macrophages in the liver to be synonymous; Kupffer cells and infiltrating macrophages express different phenotypes, and at least two subsets of macrophages have been described by Wang et al. (2014). Kupffer cell populations are unevenly distributed along the liver sinusoids, with periportal zones containing two times more Kupffer cells (43%) than midzonal and centrilobular areas (28%) of normal rat liver (Bouwens et al., 1986). The distribution gradient appears to be related to the activity of metabolic products derived from the gut microflora, which are higher in the periportal sinusoids (English et al., 2021). Kupffer cells are the principal mediators of liver inflammatory responses leading to liver injury. Kupffer cell M1 polarization stimulates the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β. Conversely, Kupffer cell M2 polarization may release anti-inflammatory cytokines like IL-10, Arg-1, and CD206 in response to NETs, contributing to the resolution of inflammation (Wang, Ma, et al., 2021; Yunna et al., 2020). CD86 serves as a marker of classically activated or inflammatory macrophage phenotype, while CD206 is a marker of alternatively activated or anti-inflammatory macrophages. In the following sections, we will review and discuss the interplay between Kupffer cells/macrophages and NETs in the modulation of liver pathophysiology.

4.1 | Kupffer cells and clearance of NETs

While NETs facilitate the extracellular killing of pathogens, excessive NET formation has been implicated in several pathological conditions. Indeed, NETs that are not sequestered from tissues or from the circulation might trigger autoimmune responses. Macrophages can remove NETs via phagocytosis. Human monocyte-derived macrophages have been shown to engulf NETs by an active, endocytic process (Farrera & Fadeel, 2013). Treatment with DNase 1 that degrades NETs facilitates NET clearance. Furthermore, internalized NETs were observed to be degraded in lysosomes. Macrophages do not produce and secrete proinflammatory cytokines IL-1β, Il-6 β, and TNFα after engulfing NETs in the absence of LPS, whereas ingestion of NETs in the presence of LPS resulted in the secretion of proinflammatory cytokines. This phenomenon implies that the clearance of NETs is not associated with an immunological mechanism, as seen in the clearance of apoptotic cells.

4.2 | NETs in macrophage polarization

The response of macrophages to neutrophils that underwent NETosis was investigated (Nakazawa et al., 2016). Macrophages (human monocytes-derived macrophages)

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were polarized to M1 and M2 phenotypes in response to NETosis induced by PMA. M2 macrophages were induced to secrete proinflammatory cytokines and chemokines—instead of anti-inflammatory factors—thereby serving as a host defense mechanism against microorganisms. Then, polarized M1 macrophages released additional extracellular DNA, which was mediated by the activity of PAD4. However, this excessive extracellular DNA was subsequently degraded by caspase-activated DNase associated with the macrophages. Thus, macrophage and NET interaction leading to clearance of excessive NETs is dependent on polarization of macrophage phenotypes, and in this case, the proinflammatory response was caused by macrophages with the anti-inflammatory M2 phenotype; however, this scenario suggests a paradoxical "repolarization" of macrophage phenotype driven by NETs (Nakazawa et al., 2016).

In SLE patients, macrophages exhibit an MI phenotype compared with healthy control cells, but in vitro differentiation abolishes this difference (Schaper et al., 2016). In culture, HMGB1, a component of NETs, promotes differentiation of macrophages into the M2 phenotype and subsequently reduces phagocytosis of apoptotic cells. The data imply that NETs modulate the phenotype differentiation of macrophages.

4.3 | NETs and macrophages in alcohol use

ALD (e.g., alcoholic hepatitis) is commonly accompanied by infiltration of neutrophils into the liver, contributing to liver injury (Cho et al., 2023; Wang et al., 2014; Zhou et al., 2018). The links between NET formation, NETosis, and NET clearance by macrophages in acute sepsis following binge alcohol drinking were studied (Bukong et al., 2018). Healthy volunteers who consumed 2 mL of vodka/kg body weight had increased blood endotoxins and spontaneous release of NETs. However, ex vivo, alcohol attenuated NET formation by neutrophils that were stimulated with PMA. Similarly, alcohol binge in mice inhibited LPS-induced neutrophil NET formation in the liver during LPS sepsis. Strikingly, acute alcohol binge significantly reduced the phagocytosis of NETotic neutrophils by the mouse macrophages, thereby impairing the clearance of NETotic neutrophils, resulting in persistent inflammation and liver injury by NETs. In addition, neutrophil depletion in vivo attenuated binge alcohol and/or LPS-induced liver damage. The findings suggest that neutrophil depletion could offer a new approach in the treatment of patients following binge drinking and associated sepsis.

In a further study, Cho et al. (2023) used co-culture of monocyte-derived macrophages and NETosis neutrophils and found that macrophages exposed to alcohol exhibited reduced phagocytosis of NETosis neutrophils compared to control macrophages not treated with alcohol. Despite reduced phagocytosis, expression of the M1 phenotype marker CD86—classically activated or inflammatory macrophage phenotype—was significantly increased in the macrophages co-cultured with NETosis neutrophils compared to control macrophages. The expression of the M2 phenotype marker CD206—alternatively activated or anti-inflammatory macrophages—remained unchanged. These data suggest that NETs can activate and induce macrophages to an inflammatory phenotype, which sustains inflammation. An interaction among alcohol, macrophages, and NETosis could promote liver damage.

4.4 | NETs induce Kupffer cells to release proinflammatory molecules

When Kupffer cells of mice cultured with conditioned media are collected from PMA-stimulated neutrophils, Kupffer cells exhibited higher levels of RNA expression of proinflammatory cytokines TNFα, IL-1β, IL-6β, and MCP-1 and chemokine CXCL10 than Kupffer cells cultured in the presence of non-stimulated neutrophils (Huang et al., 2015).

4.5 | Kupffer cells interact with NETs during acute rejection following liver transplantation

In this sterile inflammation condition, serum levels of HMGB1 and histones are elevated following the transplantation, which stimulate formation of NETs in neutrophils via the TLR4-MAPK signaling pathway (Huang et al., 2015; Liu, Yan, et al., 2022; Ni et al., 2021; Prince et al., 2011; Tadie et al., 2013), illustrated in Figure 5. In this setting, NETs promote polarization of Kupffer cells to the M1 phenotype by activating the TLR4-MAPK signaling pathway. This is followed by the translocation of HMGB1 from the nucleus to the cytoplasm and subsequent secretion into the extracellular milieu, which contributes to acute transplant rejection and aggravates liver injury, schematized below. Kupffer cell M1 polarization is characterized by increased expression of proinflammatory cytokines IL-1β, IL-6β, TNFα, and iNOS (induced nitric oxide synthetase). In addition, generation of HMGB1 by Kupffer cell MI phenotype stimulates HSC fibrogenesis via binding to the RAGE receptor of HSCs (discussed below in Section 5.2).

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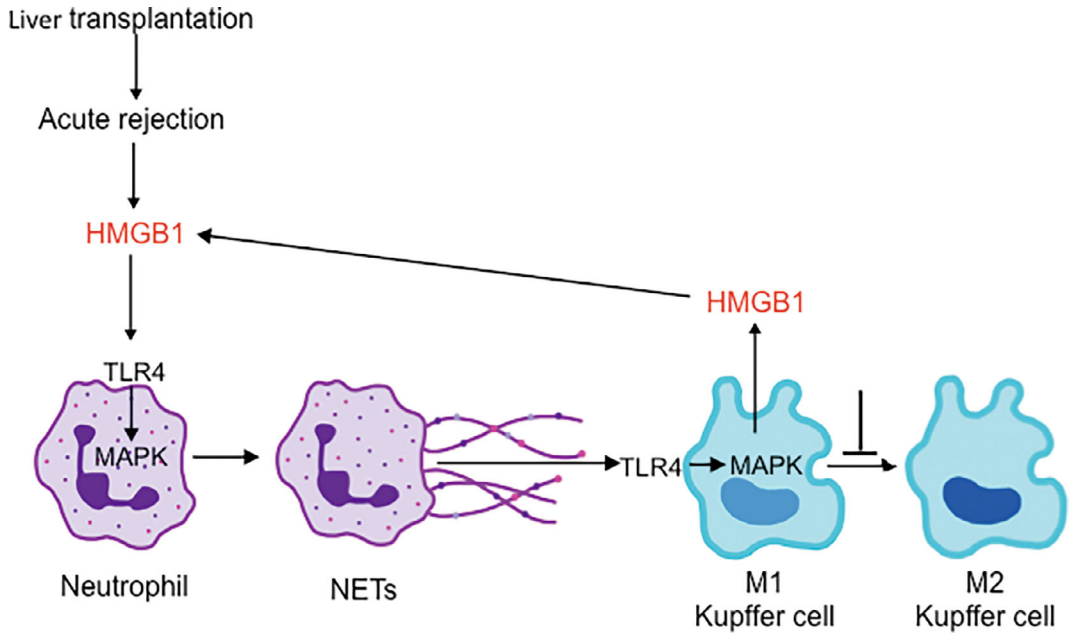

FIGURE 5 Kupffer cells interact with NETs during acute rejection following liver transplantation. HMGB1 stimulates NET release from neutrophils by activating the TLR4-MAPK signaling pathway in neutrophils. NETs in turn promote MI polarization of Kupffer cells, resulting in the secretion of HMGB1 into the extracellular milieu to aggravate liver injury.

4.6 | Kupffer cells augment neutrophil recruitment in sinusoids

LPS-induced endotoxemia in mice is accompanied by the accumulation and adhesion of neutrophils in liver sinusoids through the activation of TLR4 on neutrophils (Clark et al., 2007). The neutrophil recruitment is augmented by Kupffer cells, possibly via a distinct CD44/hyaluronan/serum hyaluronan-associated protein (SHAP)-independent mechanism in the endotoxemic livers (McDonald et al., 2013)—discussed above in Section 3.2.

In summary, proper clearance of NETs by Kupffer cells is essential for maintaining immune homeostasis and preventing excessive tissue damage. Excessive and persistent NET formation and inadequate clearance can result in chronic inflammation, leading to liver damage and fibrosis as seen in alcohol use. NETs can trap and neutralize pathogens, while Kupffer cells further process and eliminate these trapped pathogens. This cooperative interaction enhances the liver's capability to defend against infection and clear pathogens from the blood circulation. The interaction of Kupffer cells with NETs leads to enhanced release of various cytokines and chemokines. NETs can trigger a proinflammatory response in Kupffer cells, promoting MI polarization and suppressing M2 polarization. Strategies to enhance NET clearance or modulate Kupffer cell inflammation could mitigate liver damage and improve outcomes in liver disease.

5 | HEPATIC STELLATE CELLS (HSCs)

HSCs (previously called Ito cells, fat-storing cells or lipocytes) are morphologically marked by abundant vitamin A-containing cytoplasmic lipid droplets (Mak et al., 2023). HSCs reside in the space of Disse and make up 20% of the non-parenchymal cells of the liver. They are fibrogenic cells responsible for the maintenance of liver extracellular matrix structures. In response to fibrogenic stimuli (e.g., alcohol or hepatotoxins), HSCs are activated to myofibroblast-like cells to deposit excessive amounts of collagens and matrix proteins. This results in liver fibrosis. The following sections review the interaction between HSCs and NETs in liver fibrogenesis.

5.1 | Neutrophil and HSC interactions promote fibrosis in experimental steatohepatitis

Prior to our understanding of the roles of NETs in liver fibrogenesis, Zhou et al. (2018) evaluated the interplay between neutrophils and HSCs in a clinically relevant mouse model of hepatitis induced by a high-fat diet plus binge ethanol feeding. The feeding regimen induced significant neutrophil infiltration into the liver, resulting in liver injury and fibrosis. Neutrophil recruitment and infiltration into the liver were mediated by hepatic

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chemokine CXCL1 production, which is a critical step for both liver damage and liver fibrosis. Co-culture of neutrophils, but not neutrophil apoptotic bodies, with HSCs promoted HSC activation. In addition, HSCs were found to produce granulocyte-macrophage colony-stimulating factor and interleukin-15 to prolong the survival of neutrophils, creating a positive forward loop to aggravate liver fibrosis and injury damage. In addition, neutrophil-derived microRNA-233(miR-233)-enriched vesicles can be taken up by HSCs and hepatocytes, resulting in elevation of miR-233 and suppressing HSC activation and liver fibrosis via the TAZ/Indian Hedgehog (IHH) Family Zinc Finger (GLI2) and platelet-derived growth factor receptor pathways (Wang, Seo, et al., 2021). These demonstrate neutrophils crosstalk with hepatocytes and HSCs.

5.2 | Link between NETs and HSC activation with involvement of HMGB1derived from Kupffer cells and hepatocytes

NET formation by neutrophils induces Kupffer cell MI polarization. This induces secretion of HMGB1 (Liu, Yan, et al., 2022), vide supra, Section 4.5. HMGB1in turn activates HSCs via binding to the receptor for advanced glycation end products (RAGE), which leads to upregulation of the pMEK1/2/pERK1/2/pcJun signaling pathway (Ge et al., 2018; Ni et al., 2021) while increasing collagen I deposition in the liver of mice. HMGB1 signaling also stimulates the migration of HSCs. In addition, injured Kupffer cells and hepatocytes represent a major extracellular source of HMGB1 that targets the HSCs in liver fibrosis (Liu, Yan, et al., 2022). Other studies using recombinant rHMGB1 also showed that the DAMP can promote proliferation and activation of HSC T-6 cells (Ge et al., 2011; Kao et al., 2014). This scenario illustrates an interplay among HSCs, Kupffer cells, and hepatocytes in response to stimulation of NETs, effectuating HSC activation. HMGB1 is also linked to the autophagy of HSCs, as described below:

5.3 | HMGB1 triggers HSC autophagy and fibrogenesis

It is worth noting that the binding of HMGB1 to RAGE of HSCs triggers autophagy in HSCs (Li et al., 2018). The introduction of rHMGB1 to LX-2 HSCs in culture was found to elicit HSC autophagy in a dose-dependent manner, as indicated by increased LC3-II expression and decreased p62 expression. The induction of autophagy was mediated through the p-ERK/JNK MAPK pathway while suppressing the mammalian target of rapamycin (mTOR) and STAT3 pathways. Importantly, the promotion of LX-2 cell autophagy is associated with upregulation of α-SMA and an increase in collagen type 1, markers of HSC activation and fibrogenesis, respectively (Hernandez-Gea & Friedman, 2012; Lucantoni et al., 2021; Mak et al., 2023; Thoen et al., 2011).

5.4 | NETs activate HSCs via nod-like receptor protein 3 (NLRP3) inflammasome

Alcohol binges in mice fed a MASH diet (high fat-cholesterol-sugar) enhance liver fibrosis and liver injury (Babuta et al., 2024). The Nod-like receptor protein 3 (NLRP3) inflammasome complex, including caspase 1 and IL-1β, has been shown to be involved in both ALD and MASH. Overexpression of NLRP3 by activated HSCs leads to liver fibrosis (Inzaugarat et al., 2019). Alcohol binges also recruit neutrophils to the MASH liver alongside NET formation, as indicated by higher levels of hepatic NET markers cit-H3 and NE in the MASH plus alcohol binge mice. This process is mediated by an NLRP3-dependent mechanism, schematized in Figure 6.

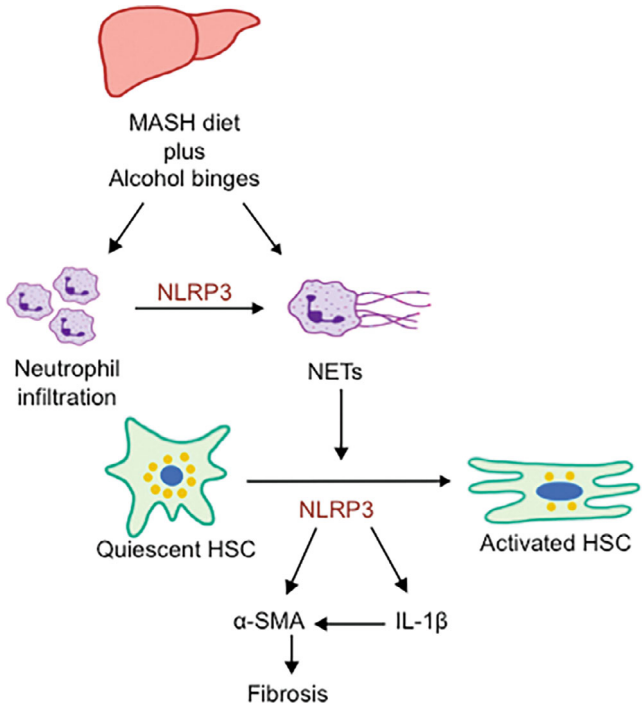

FIGURE 6 Alcohol binges in mice fed MASH diet recruits neutrophils into the liver alongside NET formation, which is mediated by a Nod-like receptor protein 3 (NLPR3)-dependent mechanism. NETs are sensed by quiescent HSCs and activate HSCs via NLRP3. NLRP3 also stimulates IL-β1 production by HSCs. Both NLRP3 and IL-β1 enhance α-SMA expression by activated HSCs, resulting in fibrosis.

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NETs also trigger HSC activation via NLRP3, which promotes IL-1β production by activated HSCs. Importantly, both NLRP3 and IL-1β enhance α-SMA expression in HSCs, resulting in fibrogenesis. The study identified a unique role for NLRP3 sensing alcohol-induced NETs, leading to HSC activation. Inhibition of NETs and/or NLRP3 may offer a therapeutic strategy to attenuate the profibrotic effects of alcohol in MASH.

5.5 | NETs and metabolic reprogramming of HSCs

The interactions between NETs and HSCs were examined in a mouse model of MASH induced by a Western diet/CCl_4 (Xia et al., 2025). Isolated primary HSCs from mice were found to highly express α-SMA in Western diet/CCl_4 compared with primary HSCs from a control normal diet. In culture, HSCs treated with NETs exhibited increased expression of α-SMA compared with controls. NETs also induced the expression of several fibrogenic genes: *Col1a1*, *Col3a1*, *Acta2*, and *Timp 1* (tissue inhibitor metalloproteinase 1). Further, NETs significantly increased HSC proliferation in a time-dependent manner after the treatment. Similarly, the migration speed and wound healing rate were markedly increased in HSCs stimulated with NETs as compared to the untreated group. In addition, NETs elevated the oxygen consumption rate, ATP-coupled respiration, and extracellular acidification rate in primary HSCs, thus upregulating ATP production from mitochondria and aerobic glycolysis to provide additional bioenergetic and biosynthetic supplies. Interestingly, NETs induced metabolic reprogramming in HSCs through their effect on arachidonic acid metabolism, which is dependent on toll-like receptor 3/cyclooxygenase-2/prostaglandin E2 pathways. Therefore, NET inhibition may offer an attractive treatment target for MASH liver fibrosis.

5.6 | NETs in liver cirrhosis

Cirrhosis is the end stage of liver fibrosis, and NETs play a significant role in the pathogenesis of liver cirrhosis by contributing to inflammation, thrombosis, and disease progression. Plasma NET levels in patients with cirrhosis were assessed (Zenlander et al., 2021). Levels of NET formation markers cit-H3 and MPO-DNA in the blood were elevated in patients with cirrhosis and correlated with the degree of liver dysfunction in patients with cirrhosis. In another study (Xing et al., 2022), the levels of NET markers MPO, NE, and cit-H3, as well as the procoagulant activity TAT complex (thrombin-antithrombin complex) in the plasma of cirrhosis patients with portal vein thrombosis (PVT), were significantly higher than those of cirrhosis patients without PVT. In addition, the levels of the NET markers correlated with the TAT complex. Therefore, inhibiting NET formation or promoting their degradation could be beneficial in preventing complications and improving outcomes in patients with liver cirrhosis.

Overall, neutrophils/NETs promote HSC activation in an animal model of steatohepatitis induced by a high-fat diet plus an alcohol model or an alcohol plus MASH model, resulting in liver fibrosis. NET stimulation of HSC activation involves the production of HMGB1, a DAMP, by Kupffer cells and hepatocytes, illustrating crosstalk among various hepatic cells. Autophagy of HSCs is triggered by NETs promoting liver fibrogenesis. NETs directly activate HSCs via NLRP3 signaling in alcohol binges plus MASH diet in mice. NETs, in a mouse model of MASH induced by a Western diet/CCl_4, elicit metabolic reprogramming of arachidonic acid metabolism in HSCs. Identification of the signaling mechanisms by which NETs mediate HSC activation offers therapeutic strategies for controlling liver fibrogenesis.

6 | HEPATOCYTES

Hepatocytes make up 70% of the liver parenchymal cell population and are the hub of intermediary metabolism of the body. Hepatocytes are responsible for various activations and detoxifications of xenobiotics, and above all, ethanol metabolism (Lieber, 1997).

6.1 | NETs and ROS in hepatocyte injury

NETs are associated with the production of ROS, which are highly reactive molecules that can interact with various cellular components and produce oxidative stress that damages hepatocytes (Jaeschke, 2000; Wang & Liu, 2021; Yao et al., 2018). ROS include superoxide anion, hydroxy radical, hydrogen peroxide, and hypochlorous acid. These are mainly produced by NADPH oxidases in a variety of cells, especially neutrophils. The mechanisms by which ROS induce hepatocyte injury are highlighted below:

1. Oxidative damage to lipids. ROS can initiate lipid peroxidation, a process whereby ROS oxidize lipids in cell membranes. The oxidation leads to the formation of malondialdehyde and other by-products that can disrupt membrane integrity, alter membrane fluidity, and impair cellular functions, including apoptosis, autophagy, and ferroptosis (Su et al., 2019).

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2. Protein oxidation. ROS can oxidize amino acid residues in proteins, leading to structural changes and affecting the functions of critical enzymes and numerous cellular processes (Cecarini et al., 2007).
3. DNA damage. ROS can cause breaks of DNA strands and/or modification of nucleotides, resulting in genomic instability and aberrant cell function, which could contribute to liver cancer (Srinivas et al., 2019).
4. Mitochondrial dysfunction. Mitochondria of hepatocytes are particularly susceptible to ROS damage. Oxidative stress can open the permeability transition pore, resulting in loss of the mitochondrial membrane permeability potential. A significant oxidant stress causes oxidation of mitochondrial NADPH and ROS generation by mitochondria—creating a vicious cycle—leading to induction of hepatocyte necrosis (Jaeschke, 2000, 2003).
5. Endoplasmic reticulum (ER) stress. ROS can induce ER stress by disrupting protein folding, leading to the accumulation of misfolded proteins. This triggers the unfolded protein response, which can affect cellular homeostasis and elicit apoptosis if the stress is severe and prolonged (Zhang et al., 2019).

7 | NETS IN HEPATOCELLULAR CARCINOMA

Hepatocytes are a source of HCC. The development of HCC is often associated with chronic liver conditions such as hepatitis B or C infection, ALD, and MASLD/MASH. These conditions can lead to chronic inflammation and liver injury, which can eventually cause genetic mutations and alterations in hepatocytes, contributing to the development of liver cancer. Experimentally, HCC can be induced by chemical carcinogens in conjunction with dietary factors. NETs have been implicated as players in cancer research, including the pathogenesis of HCC (Erpenbeck & Schon, 2017; Xin et al., 2023; Zhao & Jin, 2022; Zhu et al., 2023). Here, we review the recent laboratory and clinical investigations, linking the roles of NETs with liver tumorigenesis leading to HCC in animal models of MASLD/MASH and ALD.

7.1 | NETs promote an inflammatory environment for the development of HCC in MASH

In livers from STAM (sterile animal model)—MASH induced by neonatal streptozotocin and high-fat diet—NET formation was detected in association with infiltration of neutrophils (van der Windt et al., 2018). This was followed by an influx of monocyte-derived macrophages, production of inflammatory cytokines, and progression of MASH to HCC. Inhibition of NET formation with DNase or using PAD4-deficient mice did not affect the histogenesis of fatty liver but reduced both inflammation and tumor formation. Furthermore, MASH patients had increased serum levels of a NET marker MPO-DNA complex, reflecting the clinical relevance of the animal findings.

Wang, Zhang, et al. (2021) provided findings linking NETs with MASH-HCC from investigations using a choline-deficient high-fat diet + diethylnitrosamine (DEN) and STAM (sterile animal model) for MASH progression to HCC.

Western diets were also used to induce MASH in mice. In all these mice, neutrophil infiltration into the liver was significantly increased. There was a significant positive correlation between elevated NETs and hepatic regulatory T-cell (Treg) levels. The increase of Tregs can promote an immunosuppressive environment favoring the growth of tumor cells in the liver of MASH as well as during the transition from MASH to HCC. Importantly, NETs linked innate and adaptive immunity by promoting Treg proliferation and activity via metabolic reprogramming of naïve $CD4^+$ T cells towards mitochondrial oxidative phosphorylation (OXPHOS). The latter was mediated by the electron transport chain composed of multisubunit protein complexes (complex 1-V) on the mitochondrial membranes in a TLR-4 signaling-dependent mechanism. Therefore, the metabolic pathways controlling Treg differentiation may present potential therapeutic targets for HCC.

7.2 | NETs and LPS promote HCC in alcoholic hepatitis

Binge alcohol drinking leads to alcoholic steatosis, which is characterized by neutrophil infiltration in the liver and an increased risk of HCC. To study the potential linkages among LPS, NETs, fatty liver, hepatitis, and HCC, alcoholic steatohepatitis was induced in a chronic plus binge alcohol feeding model, wherein mice were fed an ethanol-containing liquid diet for 4 weeks, followed by three oral gavages of a single dose of ethanol (5 g/kg/day) (Liu, Zhang, et al., 2022). To study the progression of ASH to HCC, rats were injected with DEN weekly for 6 weeks to be followed by consumption of an ethanol-containing liquid diet for another 6 weeks. LPS was administered daily by subcutaneous injection. In these models, gut-derived LPS stimulated NETs via the TLR4 signaling pathway. Liver levels of NET markers MPO, NE, and histone cit-H3 were elevated, and inflammatory cytokines and chemokines (TNF-α, IL-6, CXCL-2, and

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CCL-2) were upregulated, causing hepatic inflammation after chronic alcohol consumption. These, in turn, promoted histogenesis of alcoholic fatty liver and subsequent progression to HCC. Consistent with the animal data, serum LPS concentrations and NET formation were significantly higher in patients with ALD than in nonalcoholic patients. Thus, it appears that alcohol use is linked with the formation of NETs, elaborated on below.

7.3 | Alcohol and NET formation

Alcohol can induce NETs in vivo and in vitro. Patients with alcoholic hepatitis had increased formation of NETs (Cho et al., 2023). Similarly, alcoholic hepatitis in mice demonstrated elevated NET formation. In mice, binge alcohol feeding also recruited neutrophils to the MASH liver, accompanied by increased NET formation (Babuta et al., 2024). Chronic alcohol feeding plus alcohol binges with alcoholic hepatitis induction in rats also showed elevated liver levels of NET markers—MPO, NE, and histone cit-H3 (Liu, Zhang, et al., 2022). Moreover, NETs and LPS were higher in patients with ALD plus HCC than in HCC without ALD. In vitro studies provide mechanistic links between alcohol and NET formation. NET formation was enhanced in neutrophils treated with acetaldehyde, implying that alcohol and its reactive metabolites can regulate NET formation. In addition, when aldehyde dehydrogenase was inhibited with disulfiram (FDA-approved drug for alcohol use disorder), the induction of NET formation in human neutrophils by alcohol was reduced (Babuta et al., 2024). These data are consistent with the findings of a study with a SARS-CoV-2 animal model, which showed that disulfiram inhibited NET formation (Adrover et al., 2022).

8 | NETS IN HCC METASTASIS

The involvement of NETs in the promotion of HCC has been established (vide supra). Moreover, NETs play significant roles in promoting metastasis of HCC. The initial study discovered that NETs sequester lung carcinoma cells in the circulation of hepatic sinusoids, and the trapping is associated with increased metastasis (Cools-Lartigue et al., 2013). Subsequently, the DNA component of NETs was identified in association with cancer metastasis in mouse models (Tohme et al., 2016). This reaction involves release of HMGB1 from NETs, activating TLR-dependent tumorigenic pathways in cancer cells. Using mouse models transplanted with breast or colon cancer cells of human or mouse origin, Yang, Liu, et al. (2020) showed that NETosis was induced in the livers of the mice before metastases could be detected. They identified that the DNA component of NETs (NET-DNA) was recognized by coiled-coil domain containing protein 25 (CCDC25), a transmembrane protein on cancer cells. The binding, in turn, activated the integrin-linked kinase (ILK)–β-parvin–RAC1–CDC42 cascade in the promotion of metastasis. Deletion of NETs, by either directly degrading NET-DNA using DNase I or generating PAD 4-deficient mice, an enzyme required for NETosis, significantly reduced liver metastases from mouse breast cancer cells. These data indicate that NETs play a crucial role in the process of liver metastasis from breast and colon cancer. The findings suggest that monitoring NET-DNA in blood samples could be useful in predicting the occurrence of liver metastasis.

Yang, Luo, et al. (2020) found that neutrophils isolated from HHC patients, especially those with metastatic HCC, exhibited enhanced NET formation, as measured by elevated levels of MPO-DNA and cit-H3-DNA. The increased NETs not only trapped HCC cells but also induced cell survival and enhanced invasion capacity. This triggered HCC metastatic potential, which was mediated by internalization of NETs into trapped HCC cells and activation of toll-like receptor TLR4/9–COX2 (cyclooxygenase) signaling, with enhancement of tumorous inflammatory response in the trapped HCC cells. TLR4/9 can recognize DAMPs that are present in NETs (Urban et al., 2009). Blocking TLR4/Cox2 signaling could dampen HCC metastasis triggered by NETs.

In HBV-infected HCC patients, the promotion of HCC metastasis by NETs was demonstrated both in vitro and in vivo, primarily through NETs-mediated cell entrapment and cell migration in association with epithelial-mesenchymal transition (EMT) and degradation of extracellular matrix induced by MMP (matrix metalloproteinase) (Zhan et al., 2023). Moreover, the induction of S100A9—which is a DAMP molecule—by HBV accelerated the generation of NETs through the activation of TLR4 and RAGE, along with the signaling cascade involving ROS. Furthermore, the formation of NETs in the circulation was significantly associated with TNM (tumor-node-metastasis) stage and metastasis in HBV-related HCC, and the presence of NETs was shown to have predictive value for extrahepatic metastasis.

Elevated levels of NET release have been observed in patients diagnosed with HCC, particularly those with portal vein tumor thrombosis (PVTT) (Guan et al., 2021). IL-8 produced by liver cancer cells triggers the formation of NETs via an NADPH oxidase-dependent pathway, and NET-associated cathepsin G facilitates hepatocellular carcinoma metastasis both in vitro and in vivo. Jiang et al. (2022) found that the metabolic shift of tumor-associated neutrophils towards glycolysis and the pentose phosphate

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pathway promotes the formation of NETs in a ROS-dependent manner. Subsequently, NETs stimulate tumor cell migration and suppress tight junction molecules in adjacent endothelial cells, thereby promoting tumor intravasation and metastasis. The results unveiled a pro-metastatic role of NETs in the milieu of human HCC and pointed to the importance of metabolic reprogramming in shaping their characteristics, thus providing an applicable, efficient target for anti-cancer therapies.

Neutrophils in patients with liver cancer exhibit elevated levels of mitochondrial ROS and generate NETs that are enriched with oxidized mitochondrial DNA (mtDNA) (Yang et al., 2022). When bound to NET-associated proteins, oxidized mtDNA promotes inflammation and metastasis by stimulating inflammatory mediators in HepG2 cells. Targeting oxidized mtDNA with metformin can reduce the inflammatory state that facilitates metastasis, thereby undermining the metastatic capacity of HCC.

9 | NETS IN LIVER ISCHEMIA REPERFUSION INJURY

Hepatic IRI is a major cause of liver failure and graft loss associated with liver transplantation (Liu, Yan, et al., 2022). An important component of liver IRI is sterile inflammation. Recruitment of neutrophils to liver sinusoids, their migration across the sinusoidal endothelium, and excessive activation during reperfusion contribute significantly to hepatic IRI. Specifically, NETs have been implicated in the pathogenesis of IRI. The molecules that impact NET formation and factors that prevent NET-mediated IRI are discussed in the following sections.

9.1 | Histones and HMGB1

Histones and HMGB1, commonly associated with tissue damage, are secreted by damaged hepatocytes during liver IRI. Functioning as DAMPs, histones bind TLR9, and HMGB1 binds TLR4 on the surface of neutrophils (Figure 7). The bindings activate MyD88 signaling, which then enters the nucleus to stimulate PAD4 expression and subsequent NET formation (Huang et al., 2015). The generation of NETs during liver IRI is damaging, as NETs initiate a proinflammatory response. Inhibition of NET formation by DNase1 and PAD4 inhibitor mitigates histone- and HMGB1-mediated liver IRI, respectively.

Other factors that are involved in mediating NET formation in IRI have been reported. These include the LSEC IL-33/ST2 axis (Yazdani et al., 2017), IL-17A (Tohme et al., 2019), mast cell degranulation (He et al., 2018), and TIMP-1 (Duarte et al., 2018).

9.2 | Prevention of NETs-related IRI

A study in mice has proposed that ROS generation after ischemic reperfusion activates the TLR4 signaling pathway in neutrophils and, subsequently, activates NADPH oxidase, leading to the induction of NETs (Al-Khafaji et al., 2016). Thus, inhibition of ROS by antioxidants allopurinol and N-acetylcysteine and inhibition of NADPH by DPI were shown to decrease NET formation and

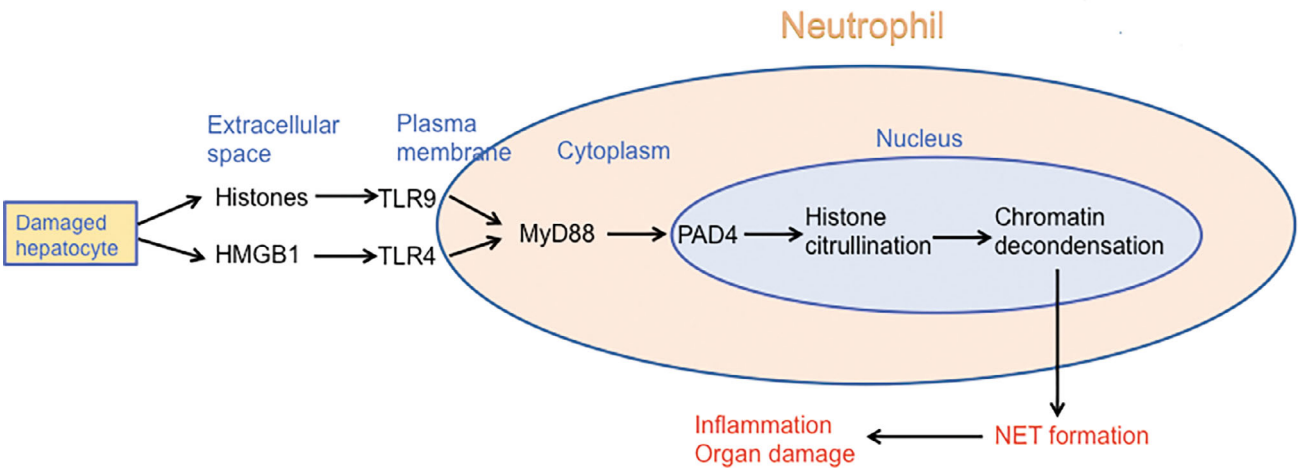

FIGURE 7 Histones and HMGB1 induction of NETs during liver ischemia reperfusion injury (IRI). During IR injury, damaged hepatocytes release histones and HMGB1. Function as DAMPs, histones, and HMGB1 engage neutrophil TLR9 and TLR4 receptors, respectively, leading to MyD88 signaling in the cytoplasm, and subsequently, PAD4 activation in the nucleus. This results in NETs release into the extracellular space causing inflammation and organ damage.

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associated liver injury after IR. This demonstrates a requirement for TLR4 and NADPH in ROS-induced NETs and also points to the role of ROS in excess of cellular antioxidant defenses in the pathophysiology of IRI. Neutrophils and NETs were found accumulated in the liver of ischemia–reperfusion mouse models, and both were negatively correlated with histidine-rich glycoprotein (HRG) expression. Supplemental HRG treatment caused decreased neutrophil infiltration and suppressed NET formation in the liver, thereby alleviating liver IRI (Guo et al., 2021). Hydroxychloroquine (anti-malarial) could diminish hepatic IRI by inhibiting NET generation via TLR9 blockade in the hepatic ischemia–reperfusion mouse model (Zhang et al., 2020). Acrolein, the end product of lipid peroxidation, can trigger NADPH oxidase and P38 MAPK signaling, causing the release of NETs in the liver during IRI, which leads to significant apoptosis and loss of mitochondrial damage in HepG2 cells (Arumugam et al., 2018). Notably, scanning electron microscopy revealed the engulfment of adhered hepatic carcinoma cells by neutrophil NETs, reflecting an induction of cell damage by NETs via direct cell-to-cell contact. Therefore, inhibition of NADPH oxidase and P38 MAPK signaling could inhibit the formation of NETs and improve the survival and functions of the post-operative liver.

Tetramethylpyrazine (TMP), a compound extracted from *Ligusticum wallichii Franchat*, has been found to mitigate hepatic IRI through inhibition of NADPH oxidase activity that mediates the formation of NETs in rats after liver transplantation (Liu, Qin, Lei, Chai, Huang, & Wu, 2021). Moreover, a synergistic effect was observed between TMP and diphenyleneiodonium, a NADPH oxidase inhibitor, in preventing hepatic IRI (Liu, Qin, Lei, Chai, & Wu, 2021). Further study found that treatment with recombinant human thrombomodulin (rTM) reduced neutrophil infiltration with lesser formation of NETs through blockade of TLR4/MAPK signaling after liver transplantation in rats (Liu, Lei, et al., 2022). Thrombomodulin is a transmembrane glycoprotein mainly expressed on the surface of endothelial cells. It plays vital roles in coagulation and inflammation and has been used to treat patients with disseminated intravascular coagulation. Collectively, these findings suggest that treatment with antioxidants can protect against liver IRI in part through attenuation of NET formation. However, the therapeutic benefits of NET inhibition should also consider the possible complications in immunocompromised individuals after transplantation.

The serine proteinase NE, a prominent component of NETs, has been implicated in the pathophysiology of IRI and hepatocyte injury (Jaeschke & Smith, 1997; Kawai et al., 2010; Otsuka et al., 2001; Takai et al., 2005). NE is a 29-kDa glycoprotein chymotrypsin-like serine proteinase stored in azurophil granules in its active form until it is released following neutrophil exposure to the inflammatory stimuli. Once released, NE is potentially fully active because it functions optimally in a neutral environment. As a result, excessive release of NE degrades elastin, basement membrane collagen, and laminins and other extracellular matrix components, leading to organ damage (Mainardi, Dixit, & Kang, 1980; Mainardi, Hasty, et al., 1980). NE also has the ability to disrupt endothelial cell adherens junctions, thereby facilitating neutrophil migration through the vessel wall (Moll et al., 1998). In addition, neutrophil NE and oxygen free radicals can induce the production of MCP-1 that in turn upregulates the expression of ICAM-1 in endothelial cells and promotes the adhesion of neutrophils, causing endothelial cell damage (Yamaguchi et al., 1999).

Hence, pretreatment with an NE inhibitor decreased neutrophil infiltration and corrected hepatocyte injury in a mouse model of IRI (Uchida et al., 2009, 2010). The expression of inflammatory cytokines TNF-α and IL-6, and chemokines CXCL1, CXCL2, and CXCL10 was significantly reduced by the neutrophil elastase inhibitors (NEI, GW311616A) or Sivelestat (for treatment of acute lung injury associated with systemic inflammation in humans). In addition, apoptosis of hepatocytes was diminished and TLR4 expression was downregulated. Thus, targeting NE offers a useful approach in preventing liver IRI.

10 | CONCLUDING REMARKS

Since the landmark discovery of NETs by Volker Brinkman and colleagues in 2004, the pivotal role of NETs in innate immunity and in infectious and noninfectious diseases, cancer, and autoimmune disorders has been established. Bibliometric analysis including a total of 4866 publications in the last two decades—2004 to 2022—reveals the trends, hot spots, and frontiers of NETs-related research (Wan et al., 2022). The analysis reveals hematology, immunology, and cell death are hot topics in this field. Hematological thrombotic disorders, sepsis, SLE, cancer, and the association of NETs with SARS-CoV-2 may be a direction for future research.

Nevertheless, controversies persist regarding the precise mechanisms underlying NET formation, and our understanding of the clearance mechanisms of NETs remains incomplete. In addition, accurately detecting or quantifying NETs presents a significant challenge due to the complexity and diversity of their morphology and composition (Stoimenou et al., 2022). Furthermore, the number/composition of molecules associated with NETs

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keeps rising. In total, 330 proteins are now identified, 74 of which have been detected in all NET preparations (Wang, Du, et al., 2024).

The liver is the largest digestive organ and performs vital physiological functions essential for the health of the body. Neutrophil infiltration and the production of NETs are highly linked with the pathogenesis of various forms of chronic liver diseases, contributing to the progression of liver damage and upsetting bodily homeostasis. Yet the impacts of NETs on the pathophysiology of individual hepatic cells have received lesser attention compared to other NET-related research in either experimental or clinical settings and have not been considered to be the trends and frontiers in NET-related research (Wan et al., 2022). Thus, the present article provides a strong and comprehensive review of data and findings in the literature—mostly in the last decade—related to the interactions between NETs and hepatic cells. LSECs, Kupffer cells, HSCs, and hepatocytes are involved in capturing neutrophils, neutrophil transmigration across the liver sinusoidal endothelium, clearance of excessive NETs, liver fibrogenesis, liver IRI, tumorigenesis, and metastasis. We also have identified interactions between Kupffer cells and LSECs in the recruitment of neutrophils and the crosstalk among Kupffer cells, hepatocytes, and HSCs in response to NET stimulation, which activates HSCs and liver fibrogenesis. However, studies are needed to examine the impacts of NETs on the capillarization of liver sinusoids and the consequential hepatic pathophysiology. Accordingly, the present review will create new directions for understanding NETs in liver disease pathogenesis and ultimately treatment.

Regarding treatment of liver injury, a number of molecules or compounds have been used to intervene in NET formation and mitigate associated liver injury after ischemic injury in mouse models. These include DNase1, PAD4 inhibitor, allopurinol, N-acetylcysteine, thrombomodulin, NADPH oxidase inhibitor DPI, herbal extract TMP, and anti-malaria drug hydroxychloroquine. Neutrophil NE inhibitors (NEI, GW311616A) or Sivelestat and non-steroidal anti-inflammatory drug aspirin have also been tested. While these compounds exhibit some positive effects against liver injury caused by NETs in animal models, their clinical applications have been limited.

NETosis, a form of cell death, has been the focus in the field of NET-associated cell death. Importantly, this mode of cell death is different from apoptosis or necrosis. However, studies on NETosis and other forms of cell death such as necroptosis or pyroptosis have been limited. Autophagy of HSCs induced by NETs has been reported. Mechanistic assessment of apoptosis, necroptosis, or pyroptosis of LSECs, Kupffer cells, HSCs, and hepatocytes in response to NETs will be novel in NETs-related cell death research going forward.

AUTHOR CONTRIBUTIONS

Ki M. Mak: Conceptualization; writing – original draft; supervision; writing – review and editing; visualization. **Aditya C. Shekhar:** Writing – review and editing; visualization. **Selena Y. Ding:** Writing – review and editing; visualization.

ACKNOWLEDGEMENTS

The authors have nothing to report.

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How to cite this article: Mak, K. M., Shekhar, A. C., & Ding, S. Y. (2025). Neutrophil extracellular traps mediate pathophysiology of hepatic cells during liver injury. *The Anatomical Record*, 1–21. https://doi.org/10.1002/ar.25673

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